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APPLICANT :	Wands et al.		
SERIAL NUMBER :	09/436,184	EXAMINER :	K. Canella
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FOR :	DIAGNOSIS AND TREATMENT OF MALIGNANT NEOPLASMS		

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#13 KD 7-28-01

July 19, 2001
Boston, Massachusetts

Assistant Commissioner for Patents
Washington, D.C. 20231

DECLARATION OF JACK R. WANDS UNDER 37 C.F.R §1.132

I, Jack R.Wands, of Waban, Massachusetts, declare and state as follows:

1. I am a co-inventor of the invention claimed in the above-referenced application and am employed by the named assignee, Rhode Island Hospital, Providence, Rhode Island.

2. I received a M.D. degree from the University of Washington in 1969 and currently serve as Chief of the Division of Gastroenterology at Lifespan Rhode Island Academic Medical Center, Director of the Liver Research Center, Professor of Medicine at Brown University School of Medicine, and Head of the Gastroenterology Section at Brown University. I am a member of the editorial boards of the academic journals Hepatology, International Hepatology Communications, Journal of Viral Hepatitis, and Viral Hepatitis Reviews, and serve as an editorial consultant for the Journal of Clinical Investigation, New England Journal of Medicine, Proceedings of the National Academy of Sciences, Journal of Infectious Disease, Gastroenterology, Journal of Virology, Virology and Nature Medicine.

3. I have been involved in research relating to cancer for over 20 years.

4. I have read the Office Action mailed on January 19, 2001 and am familiar with the Examiner's grounds of rejection of the pending claims.

5. Antisense nucleic acids, the sequences of which are complementary to 5' regulatory and 5' coding regions within exon 1 of the aspartyl (asparaginyl) beta hydroxylase (AAH) gene were made and tested to determine their ability to inhibit AAH gene expression and to inhibit tumor growth. Antisense oligonucleotides (20 mers) were designed to bind to the 5' region of the AAH mRNA and overlap with the AUG initiation codon (see attached Fig.1). The nucleotide sequence of each AAH antisense nucleic acid tested is shown in Table 1 below. The sequence of the AAH antisense oligonucleotides is complementary to sequences beginning at nucleotide 1 (Location -1), 6 (Location -6), or 11 (Location -11) upstream (prior to) the "A" of the AUG (methionine) initiation codon.

Table 1: Sequence of exemplary oligonucleotide molecules

Location (-1)
5' CAT TCT TAC GCT GGG CCA TT 3' (SEQ ID NO:10)

Location (-6)
5' TTA CGC TGG GCC ATT GCA CG 3' (SEQ ID NO:11)

Location (-11)
5' CTG GGC CAT TGC ACG GTC CG 3' (SEQ ID NO:12)

Sense
5' ATC ATG CAA TGG CCC AGC GTA A 3' (SEQ ID NO:13)

6. Using an *in vitro* cell free transcription translation assay (TNT Quick Coupled System), the human AAH cDNA (pHAAH) was used to synthesize AAH protein. *In vitro* translation was achieved with rabbit reticulocyte lysate included in the reaction mixture. The translated product was labeled with [^{35}S] methionine in the presence of reaction buffer, RNA polymerase, amino acid mixture, and ribonuclease inhibitor (RNAsin). The products were analyzed by SDS-PAGE followed by autoradiography. A luciferase (Luc) expressing plasmid was used as a positive control. Fig. 2 shows the results of an *in vitro* transcription/translation analysis of AAH antisense oligonucleotides and shows that the antisense oligonucleotides tested

block translation of the HAAH RNA and subsequent protein synthesis of HAAH protein. In the second and third lanes, synthesis of the ~85 kD AAH protein is shown (AAH, arrow) using 1 or 2 micrograms of plasmid as the template and the T7 DNA-dependent RNA polymerase primer/promoter to generate mRNA. The addition of 100x or 1000x excess antisense oligonucleotide primer resulted in progressively greater degrees of inhibition of AAH protein synthesis, whereas the inclusion of the same amounts of sense oligonucleotide had no effect on AAH protein synthesis. Fig. 3 is a bar graph showing inhibition of AAH gene expression as a result of AAH antisense oligonucleotide delivery into neuroblastoma cells.

7. Studies with central nervous system (CNS) tumor cells (Sh-SySy neuroblastoma cells) have been carried out to determine the effect of aspartyl (asparaginyl) beta hydroxylase (AAH) antisense nucleic acids inhibit expression of AAH as measured by detecting AAH transcripts and AAH gene products. Inhibition of production of AAH gene product was tested in the neuroblastoma cells. Fig. 4 shows the results of a Microtiter In situ Luminescence Quantification (MILQ) Assay and demonstrates the actual effect of the antisense oligonucleotides inside cells. Substantial reduction in HAAH gene expression was detected by simply adding the antisense oligonucleotides to the culture medium of the cells. The MILQ assay quantifies *in situ* hybridization binding in cultured cells without the need for RNA extraction. The MILQ assay was used to study competitive antisense binding inhibition to illustrate that the antisense probe hybridized to the mRNA expressed endogenously within the Sh-SySy neuroblastoma cells. In this figure, inhibition of FITC-labeled Location -6 antisense oligonucleotide binding using specific unlabeled antisense oligonucleotides is shown. Minimal inhibition of binding was observed using non-relevant oligonucleotides. The unlabeled specific oligonucleotide was capable of effectively competing for the binding site designated by the FITC-conjugated Location -6 probe, whereas the non-relevant probe exhibited significantly less inhibition at the same molar concentration. Bound probe (FITC-labeled) was detected using horseradish peroxidase conjugated antibodies to FITC, and luminescence reagents were used to detect the bound antibody. Luminescence units were corrected for cell density and are arbitrary in nature. These data indicate that cells effectively take up antisense oligonucleotides in the surrounding environment and that the oligonucleotides taken up effectively and specifically inhibit HAAH gene expression in CNS tumor cells.

8. The effect of AAH antisense nucleic acids on tumor cell growth and migration was also tested. The following cell lines were contacted with the AAH antisense nucleic acids described above: Sh-SySy neuroblastoma cells, 9L glioblastoma cells, H1 cholangiocarcinoma cells, NEC cholangiocarcinoma cells, RBE cholangiocarcinoma cells, and FOCUS hepatocellular carcinoma cells. The data indicate that the AAH antisense nucleic acids tested inhibited tumor cell growth and migration of neuroblastoma, glioblastoma, hepatocellular carcinoma, and three different cholangiocarcinoma cell types.

9. The data indicate that significant (in some cases, complete) inhibition of AAH protein synthesis was achieved with the AAH antisense oligonucleotides. Effective inhibition of gene expression in cells was observed using all three AAH antisense oligonucleotides tested. These data indicate that AAH antisense nucleic acids inhibit AAH expression in tumor cells. The data also indicate that AAH antisense nucleic acids inhibit growth of CNS tumors such as neuroblastomas and glioblastomas as well as bile duct and liver tumors such as cholangiocarcinomas and hepatocellular carcinomas.

10. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by a fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date 7/19/01

Jack R. Wands